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HUMAN CATHEPSIN D PRECURSOR IS ASSOCIATED WITH A 60 kDa GLYCOSYLATED POLYPEPTIDE

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SUMMARY Human cathepsin D is synthesized as a 53 kDa precursor. Most of it is segregated into lysosomal compartments and subjected to a proteolytic fragmentation. Using a cross-linking reagent we show that a large proportion of the precursor is associated with a distinct protein which - under denaturing and reducing conditions - is characterized as a 60 kDa glycopeptide. Studies on cells cultured in the presence of drugs known to affect the intracellular transport (deoxynojirimycin, brefeldin A and NH_4Cl) indicated that the association with cathepsin D precursor occurs early after the synthesis and is at least partially maintained after secretion.

Cathepsin D and several other soluble lysosomal enzymes are synthesized as precursors; reviewed in references (1,2). In the endoplasmic reticulum nascent cathepsin D becomes N-glycosylated which may influence its folding and the subsequent transport. Removal of glucose residues is a prerequisite for the export of the precursor from the endoplasmic reticulum (3). Phosphorylation in a cis-Golgi compartment is needed for its interaction with mannose 6-phosphate receptors in the trans-Golgi network. These receptors and alternative targeting mechanisms participate in the delivery of lysosomal enzyme precursors to lysosomal compartments. Because of the modification and segregation reactions, the precursors participate in a number of transient interactions. These might be revealed by covalently cross-linking the precursors to the interacting proteins. We show that using bifunctional reagents cathepsin D precursor can be captured in a complex with a 60 kDa glycopeptide.

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MATERIALS

Cells, culturing and metabolic labeling. Human promonocytes U937 were maintained at 37°C in a mixture of air and CO_2 (19:1) in RPMI 1640 medium (Gibco BRL, Eggenstein, FRG) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10%(v/v) foetal bovine serum (Boehringer-Mannheim, Mannheim, FRG) that had been heat-inactivated. Prior to labeling the cells were incubated with 100 nM calcitriol (1,25-dihydroxycholecalciferol, donated by Dr. M.R. Uskoković, Hoffman-La Roche, Nutley, NJ) for 3 to 4 days (4). Metabolic labeling was performed with 1.8 MBq of either [^{35}S]methionine (Amersham Buchler, Braunschweig, FRG) or a ^{35}S-labeled amino acid mixture (Tran^{35}S-label from ICN Biomedicals, Irvine, CA) in 1 ml medium containing 0.5 or 1 x 10^6 cells in a modified RPMI 1640 medium. This medium was prepared without methionine and cysteine and with 4% (v/v) dialyzed heat inactivated foetal bovine serum. Brefeldin A, a gift from Dr. A. Takatsuki (The Institute of Physical and Chemical Research, Saitama, Japan) was added at a concentration of 2.5 μg/ml at the beginning and after 4 h of labeling that was continued for another 4 h. Deoxynojirimycin, a gift from Drs. G. Scangos and D. Schmidt (Bayer AG, Wuppertal, FRG) was added 1 h prior to the labeling at a concentration of 20 mM.

Cross-linking and immunoprecipitation of cathepsin D. Prior to the cross-linking the cells were centrifuged, washed and resuspended in 1 ml 0.1 M 3-(N-morpholino)propanesulphonic acid-KOH buffer, pH 7.4. The reagents, dithiobis(succinimidylpropionate), disuccinimidyl suberate and ethylene glycolbis(succinimidylsuccinate) were added from 50 mM stock solutions in dimethylsulfoxide to a final concentration of 1 mM to either the cell suspension or the medium. These reagents were purchased from Pierce, Rockford, IL. The incubation was performed for 10 min at 37°C and was terminated by adding 0.25 ml freshly prepared mixture of 0.7 M NaCl, 0.5% sodium dodecyl sulfate, 5% Triton X-100, 2.5% Na-deoxycholate, 25 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 25 mM iodoacetamide, 5 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml DNase I and 50 mM Na-phosphate, pH 7.4. The mixture was incubated 15 min at room temperature, freeze-thawed three times and centrifuged 1 h at 40,000 xg. The supernatant was supplemented with 1.5 mM ethylenediamine tetraacetate and cathepsin D was precipitated in the presence of affinity purified rabbit anti-human cathepsin D antibody as previously described (5).

Treatment with glycosidases and separation of the labeled polypeptides. The immunoprecipitates were washed with several solutions (5) and solubilized by heating in the presence of detergents. The composition of mixtures used for the incubations with glycosidases was described previously (6). Endo-β-N-acetylglucosaminidase H and glycopeptidase F were from Boehringer-Mannheim. Prior to electrophoresis in polyacrylamide gels (5,7) the samples were heated at 95°C in the presence of 1% sodium dodecyl sulfate with or without 20 mM dithiothreitol as indicated. The gels were processed as previously (5,7).

RESULTS

U937 cells were cultured with 100 nM calcitriol to enhance the expression of cathepsin D. The cells were metabolically labeled by culturing for several hours with [^{35}S]methionine to obtain concommittant radioactive labeling of the precursor,

intermediate and large mature subunit of cathepsin D. Aliquots of the cells were treated with cross-linking reagents and the labeled proteins were extracted with a detergent mixture. Cathepsin D polypeptides were then isolated by immunoprecipitation, separated in polyacrylamide gel electrophoresis in the presence of SDS, and detected by fluorography. When the cells were treated with disuccinimidyl suberate, or when they were treated with dithiobis(succinimidylpropionate) and the electrophoresis was performed in non-reducing conditions, a major relative loss of the precursor was observed (Fig. 1, lanes 3 and 6). When the latter reagent was used and the immunoprecipitates were reduced, cathepsin D

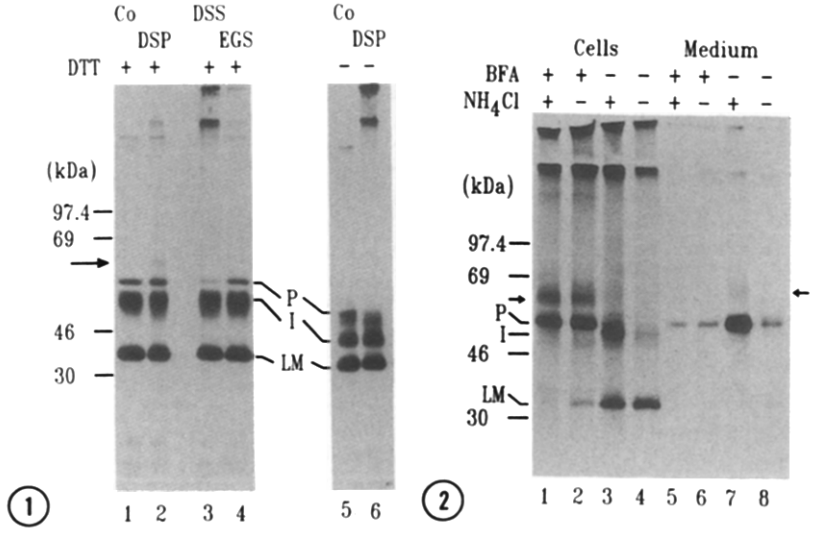

Fig. 1. Composition of the immunoprecipitates of cathepsin D from calcitriol-treated metabolically labeled U937 cells. Prior to extraction the cells were incubated for 7 min at 37°C without (Co) or with 1 mM dithiobis(succinimidylpropionate) DSP, disuccinimidyl suberate, DSS, or ethylene glycolbis(succinimidysuccinate) EGS. Cathepsin D was immunoprecipitated and the precipitates were denatured at 95°C in the presence of sodium dodecyl sulfate with or without 10 mM. The samples were either reduced or not with 10 mM dithiothreitol (DTT) and separated by polyacrylamide gel electrophoresis. The gels were processed for fluorography. The arrow indicates the material that is released from a cross-linked aggregate together with the cathepsin D precursor (P). The positions of the intermediate (I) and large mature (LM) polypeptides of cathepsin D are indicated. The standards were phosphorylase B, bovine serum albumin, ovalbumin and carbonic anhydrase.

Fig. 2. Detection of a cathepsin D precursor-binding protein by cross-linking with dithiobis(succinimidylpropionate) in cells cultured in the presence of brefeldin A (BFA) and in the medium of cells cultured in the presence of 10 mM NH_4Cl. The immunoprecipitates were reduced. The arrows indicate the position of the 60 kDa protein that is associated with cathepsin D precursor in cells and the medium. P, I and LM refer to the precursor, intermediate and large mature polypeptides of cathepsin D.

polypeptides were detected in a normal proportion to each other and were accompanied with a polypeptide with an apparent molecular mass of 58-64 kDa (Fig. 1, lane 2).

Apparently, at least a portion of cathepsin D precursor forms aggregates with other proteins. Large aggregates are obtained after cross-linking with dithiobis(succinimidylpropionate). These aggregates contain a distinct protein which is released after denaturation and reduction. It is characterized by an apparent molecular mass of 58-64 kDa and will be referred to as the 60 kDa polypeptide. In a pulse-chase experiment a transient radioactive labeling of this polypeptide was observed similar to that of the precursor cathepsin D (not shown).

Brefeldin A interferes with the vesicular transport between the endoplasmic reticulum and the Golgi apparatus (8). This drug allows for a partial phosphorylation, but it prevents the formation of "uncovered" mannose 6-phosphate residues in precursor cathepsin D and its subsequent processing as well (9). When the cross-linking with dithiobis(succinimidylpropionate) was performed with cells that had been labeled in the presence of brefeldin A, the intensity of the labeling of both the precursor cathepsin D and the 60 kDa polypeptide was enhanced (Fig. 2, lanes 1 and 2). When the labeling was performed in the presence of NH_4Cl, which is known to enhance the secretion of lysosomal enzyme precursors (7), the secreted cathepsin D precursor was accompanied by a material of somewhat larger apparent molecular mass (63-67 kDa; Fig.2, lane 7) than observed for the material that could be cross-linked to the intracellular precursor.

After a treatment of the polypeptides that were cross-linked to intracellular and secreted cathepsin D precursor with glycopeptidase F, their apparent molecular mass was reduced to approximately 55 kDa. The polypeptides that were cross-linked to cathepsin D precursor in either cells or medium were less sensitive to endo-β-N-acetylglucosaminidase H than to glycopeptidase F (Fig. 3). It is likely that the glycopeptides that are associated with precursor cathepsin D in cells and in the medium are the same polypeptides, but have different carbohydrate moieties. This could be expected for glycoproteins in which complex oligosaccharides are formed in the Golgi apparatus.

Deoxynojirimycin interferes with the deglucosylation and with the transport of precursor cathepsin D from the endoplasmic reticulum (3). If the labeling of U937 cells was performed in the

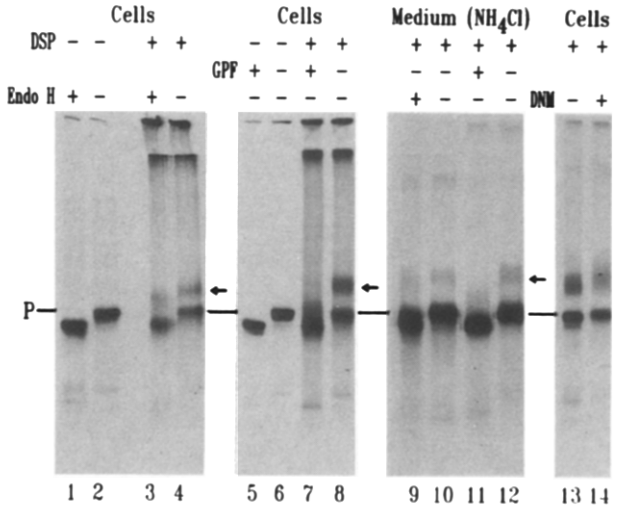

Fig. 3. Cleavage and processing of the N-linked oligosaccharides in the 60 kDa glycopeptide in calcitriol-treated U937 cells. Cathepsin D was immunoprecipitated from cells that were labeled in the presence of brefeldin A alone (lanes 1-8 and 13) and of brefeldin A and deoxynojirimycin (DNM lane 14) or from the medium of cells that were labeled in the presence of 10 mM NH_4Cl (lanes 9-12). The cells and the medium were treated with dithiobis(succinimidylpropionate) DSP as indicated. Aliquots of reduced immunoprecipitates were incubated without or with endo-β-N-acetylglucosaminidase H (Endo H) or glycopeptidase F (GPF) as indicated. The position of the 60 kDa glycopeptide in the non-digested samples is indicated by arrows.

presence of both brefeldin A and deoxynojirimycin the cross-linking resulted in a coprecipitation with precursor cathepsin D of a slightly larger glycopeptide than in the presence of brefeldin A alone (Fig. 3, lanes 13 and 14). In the presence of both drugs the precursor cathepsin D was also slightly larger indicating that the processing of both glycoproteins was inhibited in the presence of deoxynojirimycin. Thus, the less mature glycoproteins that accumulated in the presence of this drug also formed complexes and could be cross-linked.

DISCUSSION

In rabbit macrophages (10), mouse fibroblastic cells (11) and human hepatoma cells HepG2 (12) association of preferentially the precursor form of cathepsin D with membranes have been reported. These results give us no information as to whether the binding of cathepsin D precursor serves its modification, targeting, or another function. We expected that putative binding components could be detected by cross-linking to and coprecipitation with

cathepsin D in cells synthesizing cathepsin D at a high rate. These conditions are met in human promonocytes U937 cultured in the presence of calcitriol. If these cells are treated with dithiobis(succinimidylpropionate) cathepsin D precursor can be isolated by immunoprecipitation in form of large aggregates. If the aggregates are treated with a reducing agent, a 60 kDa glycopeptide is released along with precursor cathepsin D. This glycopeptide can be easily detected if the cells are metabolically labeled in the presence of brefeldin A which interferes with the intracellular protein transport (8) and induces an accumulation of cathepsin D precursor (9). The glycopeptide can also be detected in other human cells including skin fibroblasts, mammary carcinoma MCF_7, osteosarcoma MG-63 and colon carcinoma cells Caco-2 and HT-29 (not shown).

The 60 kDa glycopeptide is not cross-linked to the intermediate and mature forms of cathepsin D. During the maturation of cathepsin D its binding site or the 60 kDa glycopeptide may be destroyed. At least a portion of the 60 kDa glycopeptide is associated with precursor cathepsin D even after the secretion. After cross-linking in the medium from NH_4Cl-treated U937 cells, immunoprecipitation together with cathepsin D and reduction, the glycopeptide associated with cathepsin D has a slightly larger apparent molecular mass than after cross-linking within the cells. This and the reduction of its mass after a treatment with glycopeptidase F indicates that within the Golgi apparatus the 60 kDa glycopeptide acquires complex oligosaccharides. When the glycopeptide is isolated from cells that have been metabolically labeled in the presence of brefeldin A, and then incubated with glycopeptidase F, its apparent molecular mass is reduced to indicate the presence of approximately three N-linked oligosaccharides. Treatment with endo-β-N-acetylglucosaminidase H removes probably only one of these oligosaccharides and indicates a partial processing that may be expected after a prolonged incubation in the presence of brefeldin A (8). In U937 cells cathepsin D precursor has been shown to become partially phosphorylated but its phosphate residues have not been "uncovered" during a 4 h incubation in the presence of brefeldin A (9).

We have not examined if the cross-linked aggregates are bound to a membrane. Perhaps large aggregates of cathepsin D precursor contribute to that fraction of the precursor that after a treatment with saponin remains associated with sedimentable membranes

(11,12). However, at least in mouse fibroblastic cells, the association of cathepsin D precursor with membranes is reversible (12) so that the association with the perforated membranes cannot be explained by entrapment of the aggregates. The optimum of the binding of these precursors has been shown to occur at pH 5 (12). However, in cultured U937 cells the binding of cathepsin D precursor to the 60 kDa glycopeptide occurs (at least in the presence of brefeldin A) proximally to the Golgi apparatus and thus most probably at neutral pH. We have no data supporting the possibility that the cross-linking in U937 cells is related to an association of cathepsin D with membranes that has been described in rabbit macrophages (10). We have not been able to detect binding of cathepsin D to membranes in U937 cells using conditions of Diment et al. (10).

The 60 kDa glycopeptide may assist one of the large number of reactions from folding, carbohydrate processing, through to segregation and maturation of human cathepsin D. For the elucidation of its role we will attempt to isolate enough material for the determination of the N-terminal amino acid sequence.

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